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Intraperitoneal protein injection in the axolotl: The amphibian kidney as a novel model to study tubulointerstitial activation

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Intraperitoneal protein injection in the axolotl: The amphibian kidney as a novel model to study tubulointerstitial activation.

Background. A substantial body of experimental evidence suggests that protein loading causes activation of proximal tubular epithelial cells with consecutive interstitial fibrosis. These studies have mostly been performed using mammalian in vivo models of glomerular damage or tissue cultures of mammalian tubulointerstitial cells. The kidney of the axolotl contains not only closed nephrons, but also nephrons with ciliated peritoneal funnels called nephrostomes that have access to the peritoneal fluid. Injection of protein into the peritoneal cavity fails to expose closed nephrons to a protein load, but causes selective uptake and transient storage of proteins in tubular epithelial cells of nephrons with nephrostomes. The purpose of the present study was to determine whether (*a*) the axolotl kidney can be used as a model to assess protein uptake by tubular cells in vivo in the absence of glomerular damage, and (*b*) this is accompanied by any evidence of tubular epithelial cell activation and interstitial fibrosis.

Methods. Male and female axolotl (80 to 120 g of weight) were given a daily intraperitoneal injection of 1.5 mL endotoxin-free calf serum or saline as control. Kidneys were harvested after 4 or 10 days using perfusion fixation for light microscopy (fibrous tissue stain) and saline perfusion for immunohistochemistry (fibronectin, TGF-β and collagen I).

Results. The findings document selective storage of protein and lipids, progressive with time, in proximal tubular epithelial cells of nephrons draining the coelomic cavity. In addition, progressive focal accumulation of fibrous tissue was noted around protein-storing tubules. Immunohistochemical staining demonstrated the presence of fibronectin and TGF-β in the tubular epithelial cells and interstitial cells.

Conclusion. The axolotl kidney provides a novel in vivo model to study tubulointerstitial activation and induction of interstitial fibrosis by protein loading. The findings are independent of alterations of glomerular function that may have potential confounding effects on peritubular hemodynamics, pO_2, cell traffic, etc.

Key words: proteinuria, tubulointerstitial activation, amphibian kidney, interstitial fibrosis, TGF-β, fibronectin.

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As shown 30 years ago, renal dysfunction correlates better with expansion of the tubulointerstitial space than with glomerular damage [1–4]. Until now it has not been firmly established whether tubulointerstitial fibrosis is just a passive consequence of glomerular damage or is a player in the genesis of renal dysfunction [5]. In this context it is of interest that proteinuria per se is thought to be a major factor in the initiation and progression of renal dysfunction [6–8]. In clinical studies proteinuria is a potent predictor of filtration loss [9]. Furthermore, experimental studies clearly established that protein-loaded proximal tubular cells acquire an inflammatory phenotype, express endothelin, angiotensinogen, cytokines and the respective receptors, and synthesize extracellular matrix. Co-culture experiments showed that they also are able to activate renal interstitial fibroblasts [5]. Remuzzi, Ruggenenti and Benigni postulated interstitial activation by endothelin that is activated by high levels of protein in tubular content [6].

Such studies in mammalian systems have two potential limitations. In vitro studies face the possibility of phenotypic modulation of cells in monolayer configuration. In vivo models based on the protein overload technique [10] and glomerular damage models with proteinuric renal injury involve loss of glomerular permselectivity. Glomerular damage, however, may have repercussions on peritubular hemodynamics, peritubular oxygen tension (pO_2) or transcapillary cell traffic, and also may cause local activation of systems involved in tissue damage, such as the coagulation, the fibrinolysis, the complement and other systems.

These considerations justify interest in models of iso-

lated tubular cell protein loading in vivo in the absence of glomerular injury.

In the late thirties Randerath and others had used the amphibian kidney to document that the histological abnormalities of proximal tubular cells in proteinuric patients must be the result of increased glomerular filtration of proteins [11–14]. To this end he made use of an anatomical peculiarity of the urodelic amphibian kidney, that is, the existence of distinct nephrons that drain the peritoneal cavity by a nephrostoma. Consequently, if proteins are injected into the peritoneal cavity, protein uptake and storage is seen only in the tubular epithelial cells of these nephrons. We re-assessed this model to examine whether selective protein loading of proximal tubular epithelial cells is associated with evidence of epithelial cell activation and local interstitial fibrosis.

METHODS

The axolotl kidney

Several former studies used *Salamandra maculatum*, a close relative of the axolotl (*Amblystoma mexicanum*) [11–13]; however, because of animal protection laws, Salamandras are no longer available. Consequently, we chose the axolotl, a primitive neotenic amphibium, as the experimental animal.

The kidney of the axolotl represents an amphibian opisthonephros in which ciliated peritoneal funnels, called nephrostomes, have access to the peritoneal fluid (Fig. 1, A–D). In urodelic amphibians to which the axolotl belongs, nephrostomes connect to the proximal tubule in close vicinity to the glomerulus [15, 16]. A further peculiarity of the nephron of lower vertebrates including Amphibia is the absence of a loop of Henle so that production of hyperosmotic urine is not possible [17].

The axolotl is a well known model species for studies on neurologic and dermal pathology. The cross reactivity and specificity of a variety of human, rat and goat antibodies for transforming growth factor-β (TGF-β), fibronectin and collagen with axolotl tissue has been well documented in several studies [18–23].

Experimental protocol

Eighteen-month-old neotenic axolotls of both sexes, weighing between 80 and 120 g, were reared in the animal rooms of the Department of Zoology II, University of Karlsruhe. Eggs were obtained from the parental animals of the stock. Hatched larvae were fed with Artemia and Tubifex until they were able to take pellets of fish food.

In a pilot study kidneys of male and female axolotl differed in size and appearance, but not with respect to the presence and morphology of nephrostomes; thus, the results of male and female axolotls were pooled. The animals were maintained in tanks of aerated tap water at a constant temperature of 18°C with a 12-hour light (06.00 to 18.00 hours) and 12-hour dark cycle (18.00 to 06.00 hours).

One week prior to the study, animals were randomly allotted to two groups. The first group received daily 0.5 mL fetal bovine serum (FBS) by intraperitoneal injection (endotoxin free FBS, cc pro; S 14 M, Neustadt, Germany). The second group received 0.5 mL isotonic NaCl solution as a control.

The FBS was assured of being endotoxin free by using the Limulus amebocyte lysate assay (LAL assay; courtesy of Dr. Klaus-Peter Becker, Institute for Microbiology, Mannheim, Germany) [24]. Concentrations of oxidized lipids were not significantly higher compared to normal human serum by high-pressure liquid chromatography (HPLC; courtesy of Karin Beumann, Dept. of Pediatrics, Heidelberg, Germany) [25].

To assess uptake of FBS by proximal epithelial cells, a monoclonal antibody against bovine serum albumin was used for the immunohistological studies (αBSA; Sigma B2901, 1:25, Lot 129 H 4874, at a dilution of 1:25; Sigma Aldrich, Deisenhofen, Germany; Fig. 1F). Negative controls were performed by omitting the primary antibody (Fig. 1E).

In a pilot experiment we investigated the tubular uptake after intraperitoneal injection of human albumin (Albumin, Human, glycated A 8301; Sigma) in a concentration of 4.5 g/dL for six days. The results showed no significant differences between experimental animals and saline injected controls (data not shown).

In another pilot experiment we investigated the tubular uptake after intraperitoneal injection of FBS (endotoxin free FBS, cc pro; S 14 M) for two days. Only marginal protein accumulation was found in tubular epithelial cells in experimental animals. No interstitial fibrosis was noted.

The main study comprised two series. The first series of daily intraperitoneal injection of 0.5 mL bovine serum was terminated after four days (referred to as short-term study). In the second series, the experiment was terminated after ten days (long-term study).

Under general anesthesia (3-aminobenzotic acid ethyl ester, A-5040; 10 g/L water in the tank; Sigma) blood was obtained and retrograde perfusion was performed via the main heart ventricle. For light microscopy 3% glutaraldehyde was used as fixative. The kidneys were excised and embedded in paraffin or Epon-Araldite. For immunohistochemistry, animals were perfused with ice-cold isotonic saline. The kidneys were then excised. One part was snap-frozen and the other part was fixed with 4% formalin.

Measurement of absorbed bovine serum in amphibian blood

After the injection of FBS the serum of the axolotls yielded at best a very faint band by the Ouchterlony

double gel diffusion test (antiserum against bovine protein from Riedel-de Haën, Germany, Lot 45258; courtesy of Dr. Lohneis, Chemisches und Veterinäruntersuchungsamt Karlsruhe, Germany) [26], indicating that the peritoneal cells absorbed only a small amount of serum. The test was negative in saline-injected control animals.

Light and electron microscopy

Two-micrometer paraffin sections were stained with a connective tissue stain (Ladewig stain) and examined using light microscopy at a magnification of ×100. The tubulointerstitial changes were quantified by two "blinded" examiners who were unaware of the assignment to treatment, using a score system for each individual structural characteristic, that is, tubular dilation, protein droplets in tubular epithelial cells and interstitial fibrosis: score 0 = no change; score 1 = minimal change; score 2 = moderate change; score 3 = marked change; score 4 = very pronounced change. The tubular dilation score 0 corresponded to an average diameter of 50 μ, score 1 to 100 μ, score 2 to 150 μ, score 3 to 200 μ, and score 4 to 250 μ or more. In two randomly selected animals per group ultrathin kidney sections were cut, stained with lead citrate and uranyl acetate, and assessed using a Zeiss EM 10 at various magnifications.

Immunohistochemistry

For immunohistochemistry the following antibodies were used: anti-TGF-β (TGF-β1, polyclonal rabbit, SC-146, 1:50; Santa Cruz Biotechnology, Santa Cruz, USA), and pan-TGF-β (EO-13; 1:50; R&D Systems, Minneapolis, MN, USA), anti-fibronectin (polyclonal rabbit, F 3648, 1:200; Sigma) and anti-collagen I (rabbit α rat collagen I, AB 755, Lot 131 DDM; 1:200; Chemicon International Inc. Temecula, CA, USA). Cryostat sections of 5 μm thickness were used. The concentration that was optimal for staining with the above-mentioned antibodies was evaluated testing different dilution series in a pilot study. Negative controls were performed by omitting the primary antibody. As detection system Fast Red (K 0699; Dako, Hamburg, Germany) was used.

Glomerular, tubular and interstitial structures were assessed using a score system. Tubular epithelial cells and interstitial cells were separately quantified, evaluating the area and the intensity of staining. Two investigators who evaluated the scores were masked as to the treatment of the animals. The scores were defined as: 0 = no staining; 1 = minimal staining; 2 = moderate staining; 3 = marked staining; 4 = very pronounced staining.

Statistics

Data are given as mean ± SD. After testing for normality (Lillefors test) either the *t* test or Mann-Whitney U test was used as appropriate. The zero hypothesis was rejected at $P < 0.05$.

RESULTS

Normal anatomy of the axolotl kidney by light microscopy

In the one-year-old axolotls the kidney had a mean length of 1.45 ± 0.67 cm and a mean weight of 0.41 ± 0.05 g. Glomeruli were located in a semi-circular pattern in the kidney. The mean diameter of the glomeruli was 201 ± 23.6 μm. The unique feature was the presence of ciliated funnels, located preferentially in the cranial and middle portion of the kidney (Fig. 1). Proximal and distal tubules could easily be differentiated by the presence and absence of a brush border.

Intraperitoneal injection of bovine serum: Light and electron microscopy

Saline control axolotls. In control kidneys, almost no interstitial tissue, as defined by staining for collagen, was noted and mononuclear cells were scarce.

Axolotls with intraperitoneal protein loading. After protein loading, clusters of closely adjacent dilated tubules were seen (Fig. 2 A, B, D). The tubular lumen contained protein sludge. In the short-term experiment cell detritus was not seen in the tubular lumen, but abundant detritus was noted in the long-term experiment. The tubules containing protein sludge then comprised approximately 20 to 30% of the tubular cross sections in the cranial and middle portion of the kidney. The proximal epithelial cells were massively swollen and homogenously filled by protein droplets approximately 2 μ in diameter with no preferential location at the luminal or abluminal side, respectively. Loading with protein droplets was progressive with time, that is, more pronounced in the long-term compared to the short-term experiment. Necrosis or mitosis of tubular cells was not seen, but occasionally atrophic tubuli were noted in the long-term experiment. Distal tubules also showed protein droplets, but less than proximal tubules.

Marked accumulation of interstitial tissue around the tubules with protein droplets in epithelial cells (and sparse interstitial tissue around tubules without protein droplets) was noted in the short-term and particularly in the long-term experiment (Fig. 3). Occasionally, focal infiltrates of mononuclear cells were seen. In control animals no interstitial fibrosis was noted.

The scores of the histological parameters in the short term and the long-term experiment are given in Tables 1 and 2. The results document that the changes increased with time.

The light microscopic findings obtained with the intraperitoneal injection of bovine serum were reproduced in additional experiments in cohorts of five animals, each injected for 10 days with 1 mL of human transferrin (8 mg/mL), human low-density lipoprotein (LDL) (10 mg/mL), and human IgG (2 mg/mL), respectively.

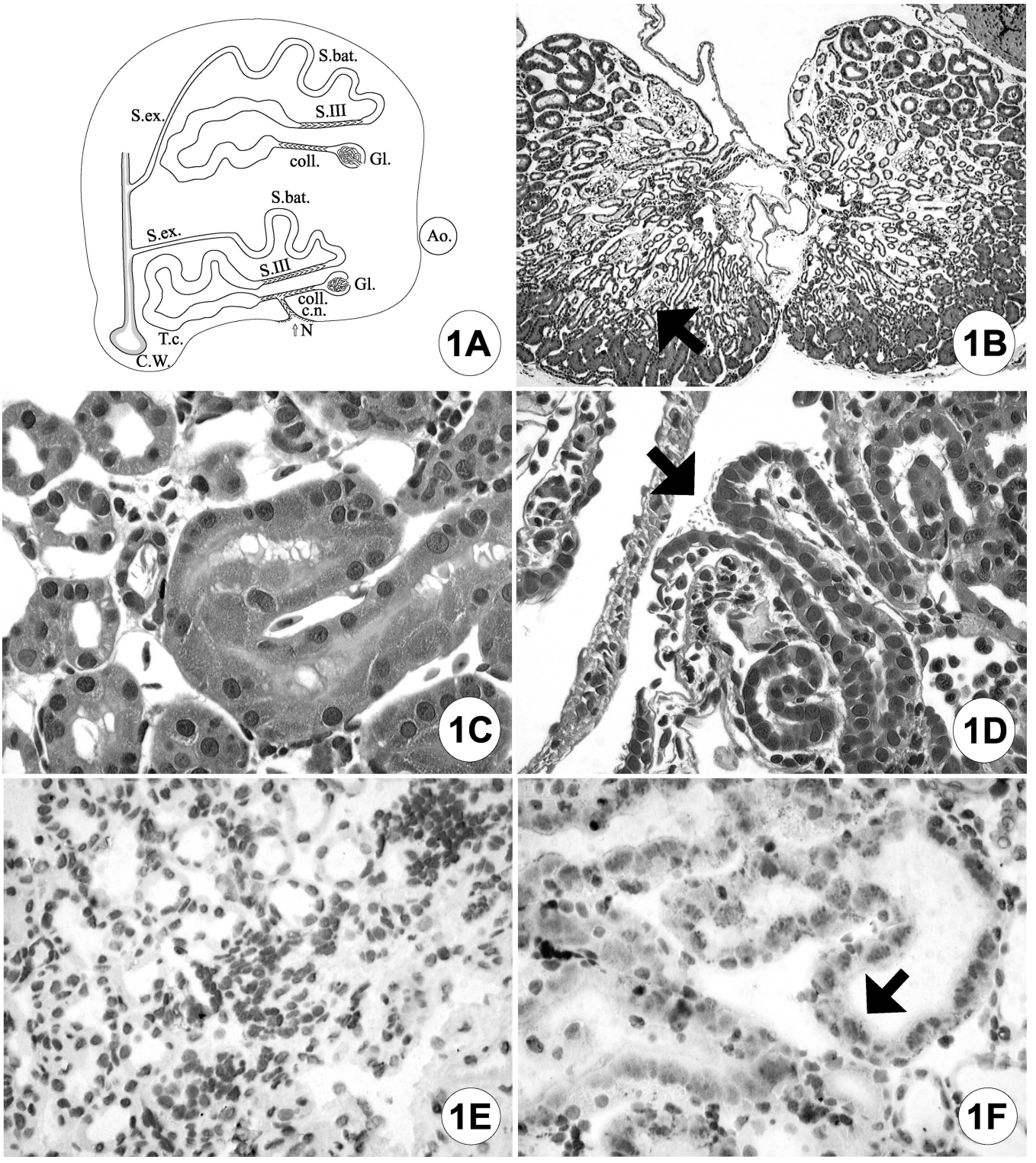

Fig. 1. (*A*) Schema of the axolotl kidney with a "closed nephron" (above) and a nephron with nephrostoma (below) [13], which drains the peritoneal cavity. The nephrostomes communicate with the peritoneum via a wide funnel decorated by long cilia (see also panel D); after Gérard and Cordier [14]. Abbreviations are: Ao, aorta; C.n., nephrostoma; coll, proximal tubule, C.W. Wolff canal; GL, glomerulus; N, funnel; S.bat, proximal part of distal tubule; SIII, Segment III corresponding part to mammalian loop of Henle; S.ex., distal part of distal tubule. (*B*) Normal axolotl kidney (saline-injected controls), low power (×40; Ladewig stain). Note subcapsular arrangement of glomeruli in one row, surrounded by distal tubules and at some distance proximal tubuli (➜) with the characteristic brush border of the epithelial cells. (*C*) Distal tubules at higher magnification (×400, Ladewig stain). (*D*) Ciliated funnel on the peritoneal surface of the cranial portion of the axolotl kidney (×400, Ladewig stain). Note cilia on the peritoneal surface and in the funnel (➜). (*E*) Axolotl, saline injection, long term experiment, high power (×400, BSA immunohistochemistry): note absence of staining. (*F*) Axolotl, protein injection, long-term experiment, high power (×400, BSA immunohistochemistry). Note marked staining of BSA positive droplets within the epithelial cells (➜).

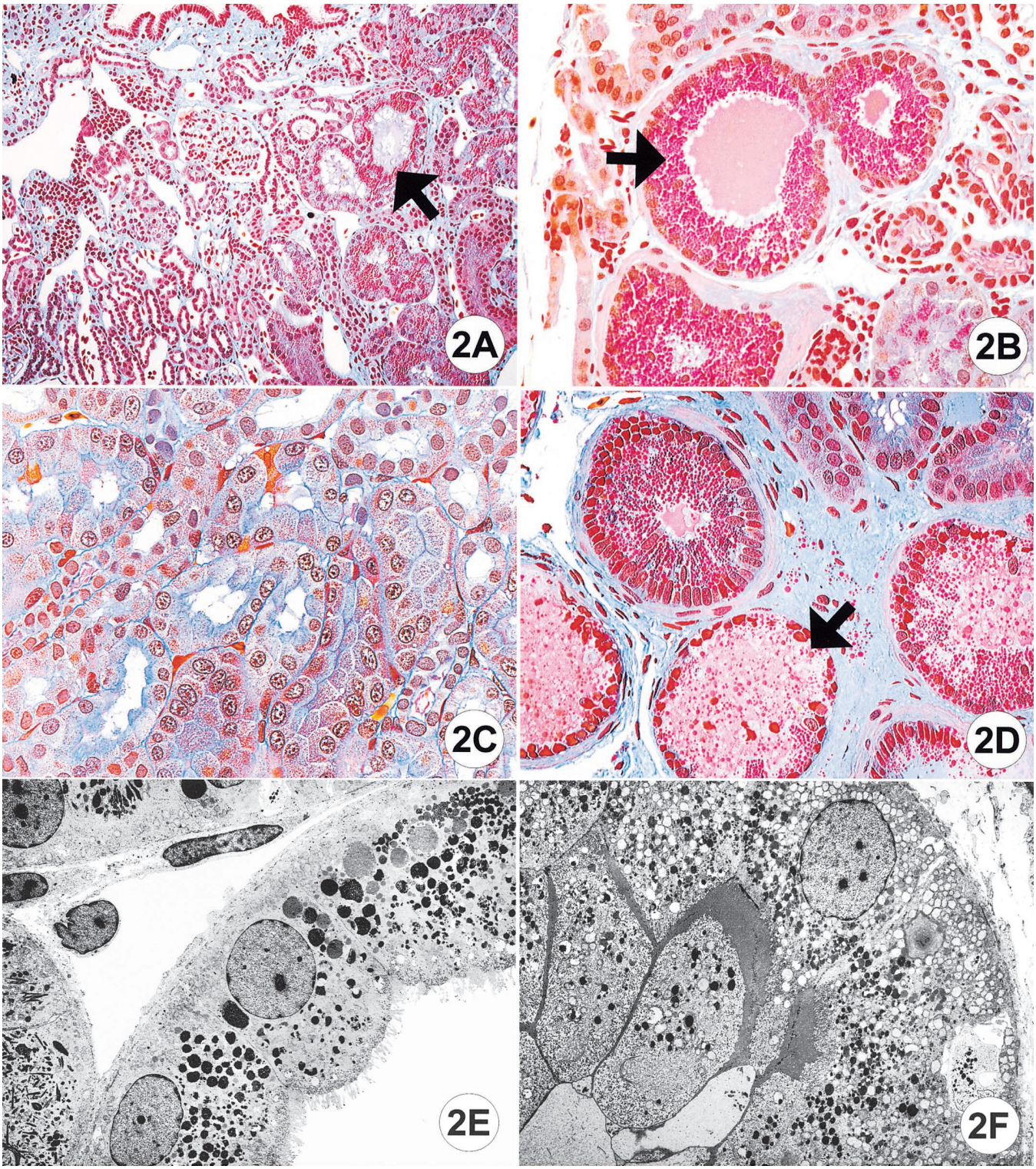

Fig. 2. Light and electron microscopy of the axolotl kidney under different experimental conditions. (*A*) Axolotl, protein injection, long-term experiment, low power (×100, Ladewig stain). At the surface (on top) one can observe ciliated funnels. Note proximal tubular epithelial cells with massive protein droplets (➜). (*B*) Protein-injected axolotl, long-term experiment, high power (×400, Ladewig stain). Note several tubules with protein-laden epithelial cells (➜) and dilated lumen filled with sludge. Note thickening of visible tubular basement membrane and staining for collagen (blue color) in the interstitium. (*C*) Axolotl, protein injection, short term experiment, high power (×400, Ladewig stain). Only delicate interstitial fibrosis is seen. (*D*) Axolotl, protein injection, long-term experiment, high power (×400, Ladewig stain). Note marked interstitial fibrosis and beginning necrosis of tubular epithelial cells (➜). (*E*) Axolotl, saline injection, long-term experiment, Electron microscopy (1700:1), proximal tubule: normal epithelial cell with brush border. (*F*) Axolotl, protein injection, long-term experiment, electron microscopy (1700:1), proximal tubule. Note enlarged epithelial cells containing numerous droplets with abundant lipids.

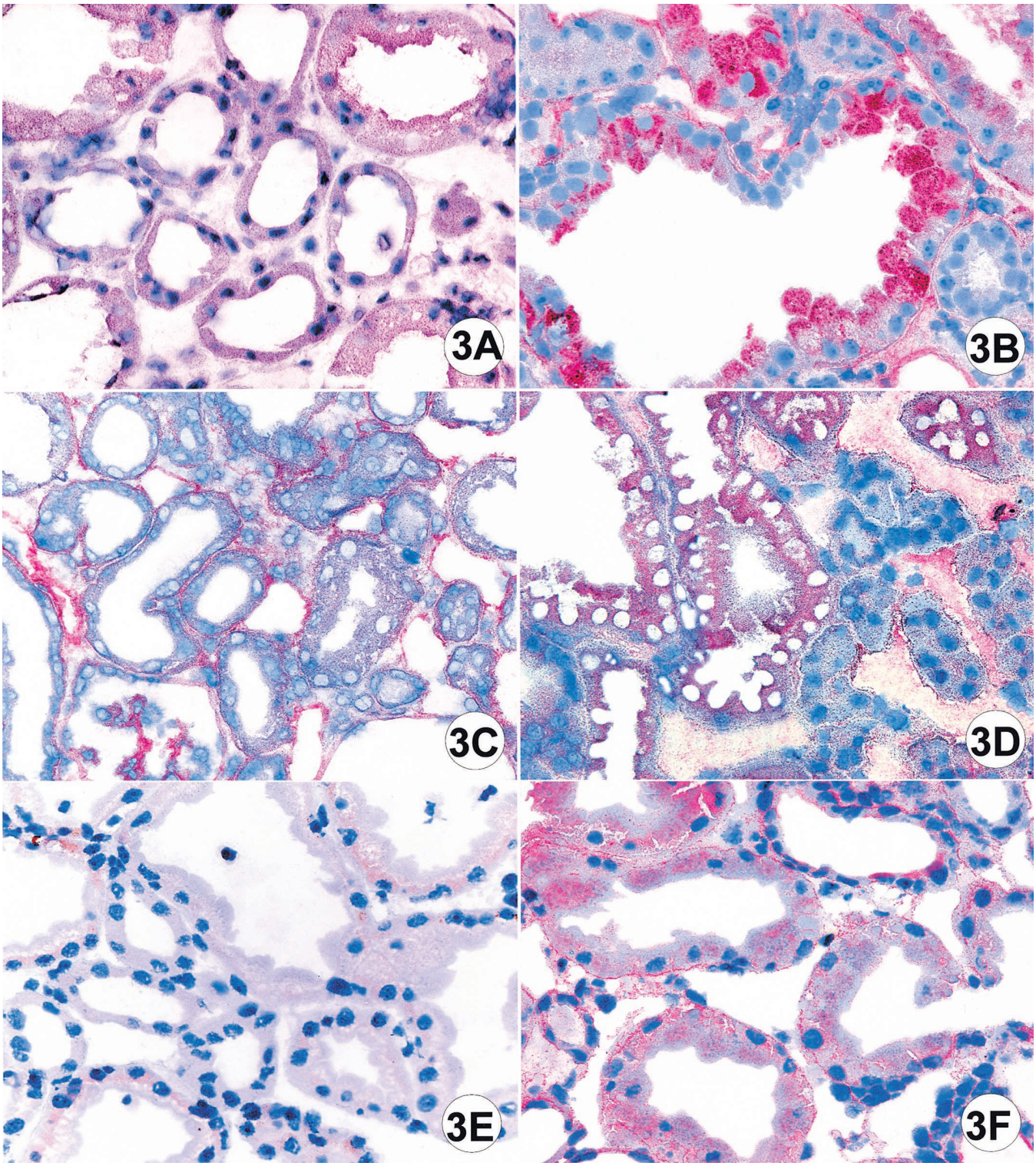

Fig. 3. Interstitial and epithelial cells after injection with saline or protein. (*A*) Axolotl, saline injection, long-term experiment, high power (×400, TGF-β immunohistochemistry). Almost no staining of tubular epithelial cells. (*B*) Axolotl, protein injection, long-term experiment, high power (×400, TGF-β immunohistochemistry). Marked staining of epithelial and interstitial cells. (*C*) Axolotl, saline injection, long-term experiment, high power (×400, fibronectin immunohistochemistry). Only minor staining of epithelial and interstitial cells. (*D*) Axolotl, protein injection, long-term experiment, high power (×400, fibronectin immunohistochemistry). Marked staining of epithelial and interstitial cells. (*E*) Axolotl, saline injection, long-term experiment, high power (×400, collagen I immunohistochemistry). No staining of epithelial and interstitial cells. (*F*) Axolotl, protein injection, long-term experiment, high power (×400, collagen I immunohistochemistry). Marked staining of epithelial cells.

Table 1. Morphological features of renal damage (short term experiment), evaluated by a score system

	Saline injected animals ($N = 5$)	Protein injected animals ($N = 5$)
Protein droplets in tubular epithelial cells	0	1.43 ± 0.42^b
Tubular dilation	0	1.49 ± 0.43^b
Interstitial fibrosis	0.26 ± 0.24	1.05 ± 0.06^a

Scores are 0 to 4 as defined in the **Methods** section.
[a] $P < 0.05$ (Mann-Whitney U)
[b] $P < 0.05$ (t test)

Table 2. Morphological features of renal damage (long term experiment), evaluated by a score system

	Saline injected animals ($N = 5$)	Protein injected animals ($N = 7$)
Protein droplets in tubular epithelial cells	1.31 ± 0.81	3.14 ± 0.54^a
Tubular dilation	0.04 ± 0.08	2.59 ± 0.86^a
Interstitial fibrosis	0.09 ± 0.17	2.36 ± 0.31^b

Scores are 0 to 4 as defined in the **Methods** section.
[a] $P < 0.05$ (Mann-Whitney U)
[b] $P < 0.05$ (t test)

Intraperitoneal injection of bovine serum: Immunohistochemistry

Two TGF-β antibodies were tested the specificity of which had been documented in earlier studies [18–23] and comparable results were found. No TGF-β staining was seen in saline-injected control animals (Table 3). In animals injected with bovine fetal serum, TGF-β staining was not seen in tubular epithelial cells that failed to exhibit protein droplets. In contrast, TGF-β immune staining was strikingly positive in some, but not all, protein-loaded proximal tubular epithelial cells. The proportion of positive tubuli was approximately 30%. The staining was specific, since no staining was seen in the negative controls without primary antibody.

In the interstitium, focally grouped interstitial cells showed strongly positive TGF-β staining in the vicinity of protein-loaded tubules. No interstitial TGF-β staining was seen in saline-injected control animals and in the interstitium surrounding tubules that failed to exhibit protein droplets. Fibronectin staining showed marked expression in the tubulointerstitial tissue in the experimental animals. In addition protein injected animals showed marked interstitial staining for collagen I.

The immunohistological finding of increased expression of pan-TGF-β, TGF-β, and platelet-derived growth factor (PDGF) expression followed by intraperitoneal injection of bovine serum was reproduced in additional experiments in cohorts of five animals, each injected for ten days with human transferrin, IgG and LDL, respectively.

Table 3. Immunohistochemical staining (long term experiment), evaluated by a score system

	Saline injected animals ($N = 6$)	Protein injected animals ($N = 6$)
Fibronectin		
Tubuli	0.90 ± 0.68	2.4 ± 0.47^a
Interstitium	1.61 ± 0.72	2.49 ± 0.49^a
TGF-β1		
Tubuli	0.34 ± 0.27	0.95 ± 0.31^a
Interstitium	0.11 ± 0.16	0.55 ± 0.32^a
Collagen I		
Interstitium	1.38 ± 0.19	2.27 ± 0.45^a

Scores are 0 to 4 as defined in the **Methods** section.
[a] $P < 0.05$ (t test)

DISCUSSION

The present study confirms for the axolotl kidney the findings obtained by Randerath [12] and others [14] in salamanders: that after injection of bovine fetal serum into the peritoneal cavity, only the nephrons that drain the coelomic cavity contain protein droplets. In Randerath's study, such selectivity was lost when low molecular weight proteins were injected that were stored in both types of nephrons, presumably because low molecular weight proteins are filtered in the glomeruli and thus reach the tubules without nephrostomes.

The novel finding in the present study is the demonstration that after daily injection of fetal bovine serum, protein-loaded tubuli not only showed luminal dilation and deposition of protein sludge, but also massive diffuse progressive accumulation of droplets containing proteins and lipids in the proximal tubular epithelial cells. This was accompanied by rapidly evolving fibrosis in the interstitium surrounding the groups of protein-loaded nephrons. Protein loading and interstitial fibrosis were further accompanied with pronounced immunohistochemical staining for TGF-β, fibronectin and collagen I. These observations are consistent with the paradigm that exposure of tubular epithelial cells to proteins causes interstitial cell activation and interstitial fibrosis in the kidney [5, 6, 27, 28].

The classical studies of Metchnikoff on phagocytosis clearly demonstrated that for specific issues, studies in non-mammalian species are more convenient than studies in mammalian species [29]. What are the potential advantages of using the axolotl kidney? We believe that the major advantage is the absence of glomerular pathology, so that artifacts resulting from confounding factors cannot occur, such as alterations of post-glomerular hemodynamics and spillover of glomerular pathology [30]. Alteration of post-glomerular pressure and flow, partial pressure of oxygen, local activation of effector systems, such as coagulation, fibrinolysis or complement system, spillover of cytokines causing cell activation and modification of trans-capillary cell traffic are excluded in this amphibian kidney model.

In view of recent interesting observations that hypoxia in the interstitium is a factor in the genesis of interstitial damage, the present model of the amphibian kidney may provide the opportunity to control for this confounding factor as well [31]. This model also excludes another interesting confounding mechanism recently proposed by Kriz et al, that of misdirected filtration [32]. According to their concept, at sites where the glomerular tuft adheres to Bowman's capsule, misdirected filtration through leaks in the basement membrane may permit filtrate to escape into the interstitium, thus causing local interstitial cell activation and fibrosis.

A distinct advantage of the present model is the life span of the axolotl, which is two to five years. This may be an advantage for long-term studies, for instance in studies on potential reversibility of interstitial fibrosis and specific aspects of its long-term evolution. It is also advantageous that many commercially available reagents showed remarkable cross-reaction with the renal tissue of axolotl [18–23]. Potential disadvantages may be the difficulty of obtaining hemodynamic measurements and the relative difficulty to obtain urine in this animal as it drains urine into a cloaca.

In the present study we tried to avoid several confounders. An effort was made to exclude the possibility that the injected FBS was contaminated by endotoxin or oxidative damage to lipids by performing the respective tests. Since we injected non-axolotl proteins, immunological reactions to FBS should not pose any problems in short-term experiments, but for long-term investigations species-specific serum protein might be preferable.

In the saline injected controls some protein droplets were seen in the long-term study. It might be that daily handling with the associated increase in the coelomic fluid by saline injection causes a moderate increase of protein in the tubular cells. Further studies must clarify the significance of this effect in long-term treated animals.

It is of note that the injected albumin was not able to induce the tubular cell damage seen with bovine serum. Further studies must be performed to clarify whether pure proteins such as IgG and transferrin are responsible for interstitial fibrosis.

The time course of protein uptake by the epithelial cells of aglomerular nephrons was remarkably rapid for a poikilotherm animal as was the appearance of peritubular fibrosis. Consequently, the model is certainly suitable for more detailed mechanistic studies.

The present study was designed as proof of the principle that the model provides useful information on tubular epithelial cell protein loading. We are aware of the nonrandom distribution of the two different nephrons and aware that in future studies more sophisticated methods of stereological analysis would be appropriate.

We conclude that the axolotl kidney provides a novel in vivo model to study tubulointerstitial activation and interstitial fibrosis by protein loading in vivo. In this model these processes occur independently of alterations of glomerular function that might have potential repercussions on the tubulointerstitial space, such as peritubular hemodynamics, pO_2 and spillover of glomerular pathology.

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